The Active Component of the Bioemulsifier Alasan from
Acinetobacter radioresistens KA53 Is an
OmpA-Like Protein

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The bioemulsifier of Acinetobacter radioresistens KA53, referred to as alasan, is a high-molecular-weight complex of polysaccharide and protein. Recently, one of the alasan proteins, with an apparent molecular mass of 45 kDa, was purified and shown to constitute most of the emulsifying activity. The N-terminal sequence of the 45-kDa protein showed high homology to an OmpA-like protein from Acinetobacter spp. In the research described here the gene coding for the 45-kDa protein was cloned, sequenced, and expressed in Escherichia coli. Recombinant protein AlnA (35.77 kDa without the leader sequence) had an amino acid sequence homologous to that of E. coli OmpA and contained 70% of the specific (hydrocarbon-in-water) emulsifying activity of the native 45-kDa protein and 2.4 times that of the alasan complex. In addition to their emulsifying activity, both the native 45-kDa protein and the recombinant AlnA were highly effective in solubilizing phenanthrene, ca. 80 μg per mg of protein, corresponding to 15 to 19 molecules of phenanthrene per molecule of protein. E. coli OmpA had no significant emulsifying or phenanthrene-solubilizing activity. The production of a recombinant surface-active protein (emulsification and solubilization of hydrocarbons in water) from a defined gene makes possible for the first time structure-function studies of a bioemulsan.

Microorganisms synthesize a wide variety of high- and low-molecular-mass bioemulsifiers (21). The high-molecular-mass bioemulsifiers, referred to as bioemulsans (20), are amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers that stabilize oil-in-water emulsions. Although most research on bioemulsans has focused on potential industrial and environmental applications (1, 9, 16, 19, 22, 28, 32), there is also a growing interest in the natural role of bioemulsans for the producing microorganism (19). To better understand how bioemulsans function in the growth and survival of microorganisms, it is essential to elucidate their detailed chemical structures as well as the genes required for their biosynthesis.

The majority of Acinetobacter strains, including both hospital and environmental isolates, produce bioemulsans (27). The best studied are the bioemulsans of Acinetobacter calcoaceticus RAG-1 (22), A. calcoaceticus BD4 (7), and Acinetobacter radioresistens KA53 (12). RAG-1 emulsan is a complex of an anionic heteropolysaccharide and protein (24). Its surface activity is largely due to the presence of fatty acids, comprising 15% of the emulsan dry weight, which are attached to the polysaccharide backbone via O-ester and N-acyl linkages (4).

The protein component of RAG-1 emulsan stimulates the emulsifying activity (34). A. calcoaceticus BD4, initially isolated by Taylor and Juni (30), produces a large polysaccharide capsule. Under certain growth conditions, the capsule is released together with the bound protein, producing a highly active

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have been defined (17, 18) should be useful for comparative studies.

MATERIALS AND METHODS

Bacterial strain and production of alasan, apoalasan, and the 45-kDa alasan protein. A. radioresistens KA53 (NCIMB-40892), isolated previously on acetate medium (12), was maintained on brain heart infusion agar (Difco Laboratories, Detroit, Mich.). After incubation for 3 days, the plates were stored at 4°C. For emulsifier production, inocula were prepared in ethanol medium (EM) containing 5 ml of ethanol, 1.8 g of urea, 13.7 g of Na2HPO4, 7.26 g of KH2PO4, 0.4 g of MgSO4·7H2O, and 1 ml of trace elements (25). Alasan production was carried out at 30°C in a 2.5-liter fermentor (Multigen; New Brunswick Scientific Co. Inc.) containing 1.4 liters of EM. Bacterial growth was initiated by introducing a 0.1% inoculum. After 80 h of batch-fed fermentation, as described previously (12), the culture reached a turbidity of 30 at 600 nm. The cell-free culture broth was then obtained by centrifugation, followed by filtration through a 0.45-μm-pore-size membrane filter. After addition of ammonium sulfate to 65% saturation, the turbid suspension was allowed to stand overnight at 4°C and then centrifuged at 10,000 × g for 20 min. The pellets were dissolved in water, dialyzed extensively against deionized water, and lyophilized, yielding 3.5 g of alasan. Apoalasan (deproteinized alasan) was obtained by the method of (35). Sodium dodecyl sulfate and 5% N-glyceraldehyde were prepared fresh by boiling at 0°C in 20 mM Tris-HCl, pH 8.5. The 4.5-kDa alasan protein was obtained from alasan by preparative SDS-PAGE as described previously (31).

Peptide analysis. SDS-PAGE was carried out on 12% gels (Bio-Rad Co., Hercules, Calif.). The gels were stained either with Coomassie blue (11) or anti-45-kDa monoclonal antibodies (10). Edman degradation analysis of the N-terminal sequence was carried out by the method developed by Qiagen (Chatsworth, Calif.). Brieﬂy, the bacterial pellet was lysed with sample buffer (0.25 M Tris [pH 6.8] and 0.8% SDS in 40% glycerol), and the lysate was incubated for 30 min with nickel beads (Qiagen) and loaded on a column. The beads were then washed with increasing amounts of imidazole (Sigma Chemical Co., St. Louis, Mo.) in order to wash out nonspecifically bound proteins. The elution of the induced protein, AlnA, was accomplished using 250 mM imidazole at a rate of 0.5 ml/min. For immunoblotting, monoclonal antibodies were produced according to a modiﬁed method of Kochel and Milstein (10).

Solubilization of PHE. To determine the kinetics of solubilization, 100 μg of PHE was crystallized in the bottom of 1-ml quartz cuvettes. The cuvettes were placed in a six-compartment holder of an Ultrascop 2000 spectrophotometer (Pharmacia, Uppsala, Sweden), and 1 ml of assay buffer containing different proteins was added to each cuvette. The amount of protein was determined by the Bradford reaction (Bio-Rad). Solubilization was performed without shaking. Absorbance readings were converted to concentration of PHE from a standard calibration curve of PHE in hexane.

Determination of emulsifying activity. A microemulsion of the standard emulsification assay (26) was used to measure emulsifying activity. Samples to be tested were introduced into 10-ml glass tubes containing TM buffer (20 mM Tris-HCl buffer [pH 7.0], 10 mM MgSO4) to a final volume of 1.5 ml, and then 0.02 ml of a 1:1 (vol/vol) mixture of hexadecane and 2-methylphosphehalene was added. The tubes were then vortexed at room temperature for 30 min. Turbidity was determined using a Gilleff spectrophotometer, model 240. One unit of emulsifying activity was defined as the amount of biopolymer that yielded an A250 of 1.0 in the assay.

Nucleotide sequence accession number. The GenBank accession number of the A. radioresistens KA53 alnA (OmpA) genomic sequence is AY033946.

RESULTS

Cloning and sequencing of the (alnA) gene coding for the 45-kDa alasan protein. Most of the emulsifying activity of the alasan complex can be attributed to the 45-kDa protein (31). Thus, its N-terminal amino acid sequence was determined. The first 19 amino acids showed 85% identity to the outer mem-

brane protein A (OmpA)-like protein of Acinetobacter spp. (15). Trypsin cleavage of the 45-kDa protein produced several internal peptides, two of which were sequenced. Each of the peptides showed a high similarity to the OmpA-like protein of Acinetobacter spp., one of them being near the C terminus. Degenerate PCR primers were designed from the N-terminal and C-terminal peptides and produced an 890-bp amplicon with genomic DNA isolated from A. radioresistens KA53. The resulting fragment was cloned and sequenced. The deduced amino acid sequence contained the three determined peptide sequences of the native 45-kDa alasan protein. This 890-bp fragment was then used to screen a mini-genomic library of strain KA53. A 2.2-kb BamHI/HindIII fragment that hybridized with the probe was extracted from the gel, cloned into pUC-18, and transformed into E. coli JM-109. Colonies were screened with the PCR primers, and several positive clones were obtained. Sequencing these clones demonstrated that the 2.2-kb fragment contained the full 1.1-kb coding sequence of the 36-kDa protein. The sequence of the first 21 amino acids upstream from the native 45-kDa protein, beginning with GVTIT, was 90% identical to the Acinetobacter sp. OmpA and 45% identical to the E. coli OmpA signal sequence. Based on the DNA sequence, the translated recombinant protein (AlnA) would contain 349 amino acids, corresponding to 37.85 kDa (35.77 kDa without the N-terminal leader sequence) with a predicted pI of 4.71. The difference in molecular mass be-

topyranoside) for 2 h, and then the recombinant protein was purified from the bacterial pellet according to the method developed by Qiagen (Chatsworth, Calif.). Brieﬂy, the bacterial pellet was lysed with sample buffer (0.25 M Tris [pH 6.8] and 0.8% SDS in 40% glycerol), and the lysate was incubated for 30 min with nickel beads (Qiagen) and loaded on a column. The beads were then washed with increasing amounts of imidazole (Sigma Chemical Co., St. Louis, Mo.) in order to wash out nonspecifically bound proteins. The elution of the induced protein, AlnA, was accomplished using 250 mM imidazole at a rate of 0.5 ml/min. For immunoblotting, monoclonal antibodies were produced according to a modified method of Kochel and Milstein (10).
tween the native 45-kDa protein and AlnA is probably due to covalent modifications of the 45-kDa protein, as discussed later. There were no cysteine codons present within the entire (1,044-nucleotide) ORF. These features are consistent with the abundant, heat-modifiable OmpA group of proteins, found in most gram-negative bacteria (3). Figure 1 shows the deduced amino acid sequence of the AlnA protein compared with that of E. coli OmpA (27% identity). The putative ATG start codon (Fig. 1) of alnA was preceded by a perfect Shine-Dalgarno sequence (GGAGGA), 5 bp upstream from the translational start codon.

Expression of alnA in E. coli. After PCR, the full-length 1.044-kb (minus the signal peptide coding sequence) gene was ligated to pET15b, a high-expression vector containing a T7 promoter and the coding sequence for an N-terminal six-His tag, forming pAT1. After transformation into E. coli, expression of the recombinant protein at 30°C following induction with IPTG was examined. Figure 2 shows that a single protein with the predicted size of 37 kDa was induced. As can be seen from the Coomassie blue-stained gel (Fig. 2), the induced protein is apparent after 0.5 h and accumulates for 2 h. The induced protein reacted with anti-45-kDa protein monoclonal antibodies. An induced 2-liter culture allowed for the purification of 25 mg of the recombinant protein, AlnA-6 His, which was used in the subsequent studies.

Emulsifying properties of recombinant bioemulsifier AlnA. A modification of the standard emulsion assay was used to determine the emulsifying activities of pure recombinant protein AlnA, the native 45-kDa bioemulsifier, alasan, and E. coli OmpA (Fig. 3). Units of emulsifying activity are defined in Materials and Methods. At 10 μg of polymer, the weight ratio of hydrocarbon to emulsifier was 1,600. The specific emulsifying activities of the 45-kDa protein, AlnA, and alasan were 179, 126, and 56 U per mg, respectively. On a molar basis, the specific activity of AlnA was 44% lower than that of the native 45-kDa protein. In all cases, emulsion turbidity was proportional to polymer concentration. E. coli OmpA showed very low emulsifying activity.

Solubilization of PHE by AlnA. Previously it was demonstrated that the alasan complex greatly enhances the aqueous solubility of polyaromatic hydrocarbons (2). To test if this property was expressed by the isolated 45-kDa alasan protein and recombinant AlnA, the solubility of PHE in quartz cuvettes was measured spectrophotometrically (Fig. 4). Without any mixing, equilibrium was obtained in ca. 3 h. The quantity of PHE solubilized by the polymers was directly proportional to their concentrations (data not presented). In the presence of...
DISCUSSION

Bioemulsans are complex mixtures of anionic polysaccharides and proteins (23). This complexity makes it difficult to analyze their chemical structures and the features required for emulsification. Once we were able to demonstrate that the surface-active component of alasan, the bioemulsan of Acinetobacter baumannii, was a protein (31), it became possible to apply techniques of molecular genetics to characterize the gene responsible for the surface activity of AlnA, which contains approximately twice the solubilizing activity of the native 45-kDa protein. Thus, on a molar basis the recombinant AlnA contains 87% of the solubilizing activity of the native 45-kDa protein.

There is significant amino acid sequence similarity (21%) between the surface-active AlnA and E. coli OmpA (Fig. 2), yet the latter showed almost no emulsifying or PHE-solubilizing activities. Thus, comparison of the structures of the two proteins might provide some insight into what structural features of AlnA allow it to be a potent emulsifier and solubilizer of hydrophobic compounds. Amino acids 6 to 171 of E. coli OmpA form a β-barrel transmembrane structure, with a hydrophilic interior and hydrophobic exterior (17, 18). Amino acids 6 to 171 of AlnA show a hydrophobicity pattern similar to that of E. coli OmpA (Fig. 5), suggesting that it might also exist as a β-barrel transmembrane structure. The striking difference between E. coli OmpA and AlnA is the high hydrophobicity of the sequences of AlnA on both ends (amino acids 2 to 10 and 168 to 175) of the presumptive β-barrel transmembrane structure. As a working hypothesis, we suggest that these two hydrophobic regions fold over the β-barrel structure, forming hydrophobic regions that are responsible for the remarkable emulsifying and PHE-solubilizing properties of AlnA. X-ray crystallography studies of AlnA are under way.

Based on its sequence similarity to other OmpA proteins, AlnA probably functions as an OmpA during exponential phase when it is cell associated (12). When growth ceases as a result of starvation or unbalanced conditions, many Acinetobacter species release bioemulsans into the medium (27). For RAG-1 emulsan, it was shown by immunological (6) and radioactive labeling (26) experiments that the source of the extracellular products is the cell membrane.

Table 1. Enhanced solubilization of PHE

<table>
<thead>
<tr>
<th>Addition</th>
<th>Solubility of PHE</th>
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<tbody>
<tr>
<td></td>
<td>µg/ml&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Buffer control</td>
<td>1.17</td>
</tr>
<tr>
<td>AlnA</td>
<td>3.54</td>
</tr>
<tr>
<td>E. coli OmpA</td>
<td>4.42</td>
</tr>
<tr>
<td>45-kDa protein</td>
<td>4.15</td>
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<tr>
<td>E. coli OmpA</td>
<td>1.34</td>
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<sup>a</sup> Solubility measurements were performed as described for Fig. 4.<br><sup>b</sup> Calculated from A<sub>252</sub> after equilibrium.<br><sup>c</sup> After subtracting solubility in the buffer control.<br><sup>d</sup> Calculated from molecular weights of PHE and the added protein. For alasan, it was assumed that the 16-, 31-, and 45-kDa proteins were present in equal molar quantities (31).
A. radioresistens.

of apoalasan and other proteins or if the complex is formed as cell surface during the growth phase. It is at present not known whether AlnA (OmpA) is the OmpA-like protein from an unidenti
derived from the sequences of genomic DNA, using probes designed from the sequences of corresponding to alasan (2). It may be that one of the functions of AlnA is to desorb, solubilize, and bring water-soluble substrates to the bacteria when they are starved of water-soluble carbon compounds. It has been reported that an

OmpA-like protein from an unidentified Acinetobacter species stimulates gastrin and interleukin-8 promoters (15). The mechanism of this promoter-stimulating activity is not known, nor is it known if the surface activity of OmpA is required for the stimulation; however, it would not be surprising to find that a water-soluble protein that binds hydrophobic compounds affects cell physiology.

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REFERENCES


FIG. 5. Hydrophobicity patterns of E. coli OmpA and A. radioresistens AlnA (OmpA). The dense alignment surface (DAS) program (5) was used to obtain hydrophobicity scores.